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(54) Soluble ligands for CD40.

(57) The present invention relates to soluble ligands for the B-cell antigen, CD40, and, in particular, to human gp39 protein and soluble ligands derived therefrom which may be used in methods of promoting B-cell proliferation.

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## 1. INTRODUCTION

The present invention relates to soluble ligands for CD40 and, in particular, to human gp39 protein and soluble ligands derived therefrom which may be used in methods of promoting B-cell proliferation.

## 2. BACKGROUND OF THE INVENTION

### 2.1. THE B-CELL ANTIGEN, CD40

CD40 is an approximately 50 kDa glycoprotein expressed on the surface of B cells, follicular dendritic cells, normal basal epithelium, and some carcinoma and melanoma derived cell lines (Paulie *et al.*, 1985, *Cancer Immunol. Immunother.*, 20:23-28; Clark and Ledbetter, 1986, *Proc. Natl. Acad. Sci.* 83:4494-4498; Ledbetter *et al.*, 1987, *J. Immunol.* 138:788-794; Ledbetter *et al.*, 1987, in "Leukocyte Typing III," McMichael, ed., Oxford U. Press pp. 432-435; Paulie *et al.*, 1989, *J. Immunol.* 142:590-595; Young *et al.*, 1989, *Int. J. Cancer* 43:786-794; Galay *et al.*, 1992, *J. Immunol.* 149:775). Isolation of a human cDNA encoding CD40 showed that this protein is a type I membrane protein which is significantly related to the members of the nerve growth factor receptor family (Stamenkovic *et al.*, 1989, *EMBO J.* 8:1403-1410).

The role of CD40 in B cell activation is well established. Crosslinking CD40 with anti-CD40 monoclonal antibodies (mAb) induces B cell aggregation via LFA-I (Gordon *et al.*, 1988, *J. Immunol.* 140:1425-1430; Barrett *et al.*, 1991, *J. Immunol.* 146:1722-1729), increases serine/threonine (Einfeld *et al.*, 1988, *EMBO J.* 7:711-717) and tyrosine (Uckun *et al.*, 1991, *J. Biol. Chem.* 266:17478-17485) phosphorylation of a number of intracellular substrates, and provides a "competency" signal which allows B cells to proliferate and undergo class switching when stimulated with the appropriate second signal. For example, anti-CD40 mAb can synergize with phorbol myristyl acetate (PMA; Gordon *et al.*, 1987, *Eur. J. Immunol.* 17:1535-1538) or anti-CD20 Mab (Clark and Ledbetter, 1986, *Proc. Natl. Acad. Sci.* 83:4494-4498) to induce B cell proliferation, with IL-4 to induce B cell proliferation (Gordon *et al.*, 1987, *Eur. J. Immunol.* 17:1535-1538; Rousset *et al.*, 1991, *J. Exp. Med.* 173:705-710) and IgE secretion (Jabara *et al.*, 1990, *J. Exp. Med.* 172:1861-1864; Rousset *et al.*, 1991, *J. Exp. Med.* 173:705-710; Gascan *et al.*, 1991, *J. Immunol.* 147:8-13; Zhang *et al.*, 1991, *J. Immunol.* 146:1836-1842; Shapira *et al.* 1992, *J. Exp. Med.* 175:289-292) and with IL-10 and TGF- $\beta$  to induce IgA secretion by sIgD<sup>+</sup> B cells (DeFrance *et al.*, 1992, *J. Exp. Med.* 175:671-682). Also, there is evidence that CD40 delivered signals are involved in modulating cytokine production by activated B cells (Cairns *et al.*, 1988, *Eur. J. Immunol.* 18:349-353; Clark and Shu, 1990, *J. Immunol.* 145:1400-1406).

Crosslinking of anti-CD40 mAb alone is not sufficient to induce B cell proliferation as demonstrated by the observation that anti-CD40 mAb immobilized on plastic in conjunction with IL-4 is unable to induce vigorous B cell proliferation (Banchereau *et al.*, 1991, *Science* 251:70-72). However, anti-CD40 mAb immobilized on murine L cells transfected with an Fc receptor, CDw32, are able to induce B cell proliferation in the presence of IL-4 (Banchereau *et al.*, 1991, *Science* 251:70-72), suggesting that a signal provided by the fibroblasts synergizes with the CD40 signal and IL-4 to drive B cell proliferation.

### 2.2. THE T-CELL ANTIGEN, GP39

Soluble forms of the extracellular domain of human CD40 such as CD40-Ig have been used to show that the CD40 ligand, gp39, is a glycoprotein of approximately 39 kDa expressed on the surface of activated CD4<sup>+</sup> murine T cells (Armitage *et al.*, 1992, *Nature* 357:80-82; Noelle *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:6550-6554). Interaction with gp39 induces resting B cells to enter the cell cycle and become responsive to the growth and differentiation effects of lymphokines (Armitage *et al.*, 1992, *Nature* 357:80-82; Noelle *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:6550-6554).

Recently, a cDNA encoding murine gp39 has been isolated and shown to be functionally active when expressed as a membrane protein on transfected cells (Armitage *et al.*, 1992, *Nature* 357:80-82). This cDNA encodes a 260 amino acid polypeptide with the typical features of a type II membrane protein and CV1/EBNA cells expressing murine gp39 were shown to induce murine and human B cell proliferation without additional co-stimulus.

## 3. SUMMARY OF THE INVENTION

The present invention relates to soluble ligands for CD40, and, in particular, to human gp39 protein and soluble ligands derived therefrom. It is based, at least in part, on the discovery, cloning, and expression of

the human T cell antigen gp39, a ligand for the CD40 receptor. It is also based, in part, on the preparation of a soluble form of human gp39 which, together with a co-stimulating agent, is able to promote B cell proliferation and differentiation.

The present invention provides for essentially purified and isolated human gp39 protein having a sequence substantially as set forth in Figure 1, as well as for essentially purified and isolated nucleic acid having a sequence substantially as set forth in Figure 1 and/or encoding said human gp39 protein.

The present invention further provides for soluble forms of human as well as non-human gp39. In a preferred, non-limiting embodiment of the invention, soluble gp39 may be produced using the expression vector CD8-gp39.

The soluble gp39 of the invention may be used, together with co-stimulating agents, to promote the proliferation of B-cells *in vivo* or *in vitro*. Such proliferation may be desirable in the treatment of conditions that would benefit from an augmented immune response, such as acquired immunodeficiency syndrome or for the generation of a cell culture system for long-term B-cell growth.

#### 4. DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide and predicted amino acid sequence of human gp39 and homology to murine gp39, TNF $\alpha$  and TNF $\beta$ .

(A) The nucleotide sequence [SEQ. ID NO: 1] and translated open reading frame [SEQ. ID NO: 2] are numbered at left. Sites of potential N-linked glycosylation are marked (CHO), the predicted transmembrane domain (TM) is underlined and the two Arg residues located at the junction of the predicted transmembrane and extracellular domains are double underlined. Nucleotide and amino acid numbering is given to the left.

(B) Alignment of the predicted amino acid sequence of human gp39 (H-gp39) [SEQ. ID NO: 3], murine gp39 (M-gp39) [SEQ. ID NO: 4], human TNF $\alpha$  (H-TNF $\alpha$ ) [SEQ. ID NO: 5], and human TNF $\beta$  (H-TNF $\beta$ ) [SEQ. ID NO: 6]. Amino acids shared by at least three proteins are shown boxed; similar amino acids shared by at least three of the proteins are shown shaded.

Figure 2. Soluble recombinant human gp39 and CD72, sgp39 and sCD72. (A) The cDNA fragment encoding the extracellular domain of murine CD8 is designated mu-CD8 EC. The murine CD8 amino terminal secretory signal sequence is shown stippled. The cDNA fragment encoding the extracellular domain of human gp39 or CD72 are designated hu-gp39 EC and hu-CD72 EC, respectively. The amino acid sequences predicted at the site of fusion of the extracellular domain of murine CD8 and human gp39 [SEQ. ID NO: 7] (*italic*) or CD72 [SEQ. ID NO: 8] (*italic*) are shown below the individual diagrams. Residues introduced at the junction of the two cDNA fragments are shown underlined. The unique Bam HI restriction enzyme recognition site at the junction of the two genes is shown. (B) Radiolabelled proteins from the supernatants of metabolically labeled mock (lanes 1 and 2) of CD8-gp39 (lanes 3 and 4) transfected COS cells were immunoprecipitated based on their interaction with the anti-murine CD8 mAb 53-6 (lanes 1 and 3) or the CD40-Ig (lanes 2 and 4) and analyzed by SDS-PAGE under reducing conditions as described in the text. The electrophoretic mobility of molecular mass standards of the indicated mass in kDa are shown to the left. (C) Radiolabelled proteins from the supernatants of metabolically labeled mock (lanes 1-4) and CD8-CD72 (lanes 5-8) transfected COS cells were recovered based on their reactivity with the anti-murine mAb 53.6 (lanes 1 and 5), the anti-CD72 mAb J3101 (lanes 2 and 6), the anti-CD72 mAb BU41 (lanes 3 and 7) and CD40-Ig (lanes 4 and 8) and analyzed by SDS-PAGE under reducing conditions as described in the text. The electrophoretic mobility of molecular mass standards of the indicated mass in kDa are shown to the left.

Figure 3. Binding of sgp39 or CD40-Ig to transfected COS cells. COS cells transfected with either a gp39 (A and B) or a CD40 (C-F) cDNA expression plasmid were examined for their ability to bind either soluble recombinant CD40 (A and B), or soluble recombinant gp39 (C and D), or the anti-CD40 mAb G28-5 (E and F) as described in the text. Phase (A, C and E) and fluorescent (B, D and F) images of representative fields are shown.

Figure 4. Characterization of the sgp39/CD40-Ig interaction. The ability of increasing concentrations of CD40-Ig (0.6  $\mu$ g/ml to 20  $\mu$ g/ml) and the control immunoglobulin fusion protein, Leu8-Ig (0.6  $\mu$ g/ml to 20  $\mu$ g/ml), to bind to immobilized sgp39 was examined by ELISA as described in the text. Likewise the ability of increasing concentrations of CD40-Ig to bind to the immobilized control fusion protein sCD72 was also examined in the same way. In both cases the sgp39 and sCD72 were immobilized on plastic which had been previously coated with the anti-murine CD8 mAb 53-6 as described in the text.

Figure 5. Activation of human B cells by surface bound gp39. The ability of gp39-expressing COS cells (gp39-COS) or mock transfected COS cells (mock COS) to stimulate the proliferation of resting human

peripheral blood B cells alone or in the presence of the anti-CD20 mAb IF5 (+ IF5) or PMA (+ PMA) in the absence (solid bars, alone) or presence (hatched bars, + CD40-Ig) of CD40-Ig was examined as described in the text and evaluated by [<sup>3</sup>H]-thymidine incorporation.

Figure 6. Activation of human peripheral blood B cell by sgp39. The ability of soluble recombinant gp39 (sgp39, hatched bars) or control soluble recombinant fusion protein (sCD72, solid bars) to stimulate the proliferation of resting human peripheral blood B cells alone or in conjunction with the anti-CD20 mAb IF5 (+ IF5) or PMA (+ PMA) was examined as described in the text, evaluated by [<sup>3</sup>H]-thymidine incorporation and compared to that of B cells incubated for an equivalent amount of time in the absence of exogenous stimuli (cells alone, open bars) or in the presence of either IF5 alone or PMA alone (open bars).

Figure 7. Activation of dense human tonsillar B cells by sgp39. The ability of soluble recombinant gp39 (sgp39, hatched and solid bars) to stimulate the proliferation of dense tonsillar B cells alone or in conjunction with the anti-CD20 mAb IF5 (+ IF5) or PMA (+ PMA) was examined as described in the text, evaluated by [<sup>3</sup>H]-thymidine incorporation and compared to that of B cells incubated alone (cells alone, open bars) or in the presence of either IF5 alone or PMA alone (open bars). The ability of CD40-Ig (solid bars) to block the sgp39 driven B cell activation was examined at a concentration of 20 mg/ml (A) and compared to an equal concentration of an irrelevant immunoglobulin fusion protein, Leu-8-Ig (solid bars, B).

Figure 8. Amino acid [SEQ. ID NO: 9] and nucleic acid [SEQ. ID NO: 10] sequence of murine CD8.

Figure 9. Amino acid [SEQ. ID NO: 11] and nucleic acid [SEQ. ID NO: 12] sequence of human CD8.

## 5. DETAILED DESCRIPTION OF THE INVENTION

For clarity of description and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) cloning and expression of human gp39 (hgp39);
- (ii) preparation of soluble gp39 (sgp39); and
- (iii) utility of the invention.

### 5.1. CLONING AND EXPRESSION OF HUMAN GP39

The present invention provides for essentially purified and isolated nucleic acids encoding hgp39, for essentially purified and isolated hgp39 protein, and for methods of expressing hgp39. The complete nucleic acid sequence of hgp39 (corresponding to cDNA) and the complete amino acid sequence of hgp39 are presented in Figure 1 and contained in plasmid CDM8-hgp39, deposited with the American Type Culture Collection (ATCC) as *Escherichia coli*, CDM8 MC1061/p3-hgp39 and assigned accession No. 69050. An example of an expression vector that may be used to produce soluble hgp39 (shgp39) is plasmid CDM7B<sup>-</sup>-shgp39 which has been deposited with the ATCC as *Escherichia coli* CDM7B<sup>-</sup> MC1061/p3-shgp39 and assigned accession number 69049.

In particular embodiments, the present invention provides for an essentially purified and isolated nucleic acid having a sequence substantially as set forth in Figure 1, and for an essentially purified and isolated nucleic acid encoding a protein having a sequence substantially as set forth in Figure 1. The present invention further provides for an essentially purified and isolated protein having a sequence substantially as set forth in Figure 1.

The term "substantially", as used herein, indicates that the sequences set forth in Figure 1 may be altered by mutations such as substitutions, additions, or deletions that result in a molecule functionally equivalent to a protein having a sequence as set forth in Figure 1. For example, due to the degeneracy of the genetic code, the nucleic acid sequence as set forth in Figure 1 may be altered provided that the final sequence encodes a protein having the same sequence as depicted in Figure 1 or a functionally equivalent sequence; i.e., an amino acid sequence in which functionally equivalent amino acids, such as amino acids of the same group (e.g. hydrophobic, polar, basic, or acidic) are substituted into the protein.

For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. The proteins of the invention may also be differentially modified during or after

translation, e.g. by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, etc.

Genomic or cDNA clones containing hgp39-encoding sequences may be identified, for example, by synthesizing oligonucleotide probes that contain a portion of the hgp39 sequence depicted in Figure 1, and using such probes in hybridization reaction by the method of Benton and Davis (1977, *Science* 196:180) or Grunstein and Hogness (1975, *Proc. Natl. Acad. Sci. U.S.A.* 72:3961-3965). Similarly, oligonucleotide primers containing a portion of the hgp39 sequence depicted in Figure 1 may be prepared and used in polymerase chain reactions (Saiki et al., 1985, *Science* 230:1350-1354), using, for example, cDNA from activated T lymphocytes as template, to generate fragments of hgp39 sequence that may be pieced together to form or otherwise identify a full-length sequence encoding hgp39.

In a specific, non-limiting embodiment of the invention, cDNA encoding hgp39 may be isolated and characterized as follows. CD40-Ig, as described in Noelle et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6550-6554, may be modified by the introduction of three mutations, namely L234F, L235E, and G237A, in the immunoglobulin domain, which reduce the binding to Fc receptors. The modified CD40-Ig may be purified from COS cell supernatants as described in Aruffo, 1990, *Cell* 61:1303-1313. Human gp39 cDNA may be amplified by polymerase chain reaction (PCR) from a library prepared from phytohemagglutinin-activated human peripheral blood T-cells (Camerini et al., 1989, *Nature* 342:78-82). The oligonucleotide primers may be designed based on the sequence of murine gp39 (Armitage et al., 1992, *Nature* 357:80-82) and may be engineered to include cleavage sites for the restriction enzymes XbaI and HindIII, to be used in subcloning the PCR product. For example, and not by way of limitation, the following oligonucleotides may be used: 5'-GCG AAG CTT TCA GTC AGC ATG ATA GAA ACA-3' [SEQ. ID NO: 13] and 5'-CGC TCT AGA TGT TCA GAG TTT GAG TAA GCC-3' [SEQ. ID NO: 14]. Amplification may be performed with Tag polymerase and the reaction buffer recommended by the manufacturer (Perkin Elmer Cetus Corp., Norwalk, CT) using 30 cycles of the following temperature program: 2 min., 95 °C; 2 min., 55 °C; 3 min., 72 °C. The PCR product may be digested with HindIII and XbaI and should be found to contain an internal HindIII restriction site. The resulting HindIII-XbaI fragment may then be subcloned into a suitable vector, such as, for example, the CDM8 vector. The complete gene product may be constructed by subcloning the HindIII-HindIII fragment into the vector containing the HindIII-XbaI fragment. The resulting construct may then be transfected into COS cells using DEAE-dextran as described in Aruffo et al., 1990, *Cell* 61:1303-1313. Transfectants may be stained with CD40-Ig (25 µg/ml in DMEM media) followed by FITC-conjugated goat anti-human IgG Fc antibody (1:50 dilution in DMEM, TAGO, Burlingame, CA) and visualized by immunofluorescence microscopy. A clone containing the complete hgp39 sequence may be obtained by colony hybridization as described in Sambrook et al., 1989, in "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, NY. The subcloned HindIII-HindIII fragment of the PCR product may be used to generate a <sup>32</sup>p-labelled probe by random primed polymerization. Plasmid DNA from several individual clones may be transfected into COS cells and the transfectants may be stained with CD40-Ig. Clones that give rise to positive-staining COS cell transfectants may then be further characterized by restriction fragment mapping and sequencing.

Once obtained, the hgp39 gene may be cloned or subcloned using any method known in the art. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids, or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, puC, or Bluescript™ (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

The hgp39 gene may be inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequence are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified.

In order to express recombinant hgp39, the nucleotide sequence coding for hgp39 protein, or a portion thereof, may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted peptide/protein encoding sequence. The necessary transcription and translation signals can also be supplied by the native hgp39 gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include, but are not limited to, mammalian cell systems infected with virus (e.g. vaccinia virus, adenovirus, etc.) or transfected with plasmid expression vector; insect cell systems infected with virus (e.g. baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities.

Expression of nucleic acid sequence encoding hgp39 protein or a portion thereof may be regulated by a second nucleic acid sequence so that hgp39 protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of hgp39 may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control hgp39 expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the cytomegalovirus promoter, the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797); the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731); promoter elements from yeast or other fungi such as the Gal 4 promoter or the alcohol dehydrogenase promoter; and animal transcriptional control regions, such as the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), the beta-globin gene control region which is active in myeloid cells (Magram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94), and other tissue-specific or constitutive promoter/enhancer elements.

Recombinant hgp39 protein or peptide expressed in such systems may be collected and purified by standard methods including chromatography (e.g. ion exchange; affinity (for example, using CD40 as ligand); and sizing column chromatography) centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

According to the present invention, hgp39 protein or peptide may also be synthesized chemically using standard protein synthesis techniques.

## 5.2. PREPARATION OF SOLUBLE GP39

The present invention also provides for soluble forms of gp39, including both human and non-human gp39. Such soluble forms of gp39 are produced by genetic engineering of gp39-encoding nucleic acid, such as hgp39-encoding nucleic acid (see Section 5.1, *supra*, and Figure 1), or Murine gp39-encoding nucleic acid (Armitage et al., 1992, Nature 357:80-82), to produce gp39 fusion proteins which comprise the extracellular domain of gp39, which extends from about amino acid residue 48 to amino acid residue 261. In addition to gp39 amino acid sequence, the fusion proteins of the invention may further comprise a molecular "tag", which may be a portion of a larger protein and which replaces the transmembrane and cytoplasmic domains of gp39 and provides a "handle" that reacts with reagents. Soluble gp39 may also be prepared without a "tag" by replacing the cytoplasmic and transmembrane domain of gp39 with an amino terminal signal peptide derived from a type I membrane protein or a secreted protein.

Because gp39 is a type II membrane protein and is therefore oriented with a carboxy-terminal extracellular domain, the tag is desirably oriented amino-terminal to the gp39 extracellular domain (gp39 ECD). Preferably, the tag peptide contains an amino-terminal secretory signal sequence to allow export of the fusion protein.

Appropriate tag proteins include extracellular protein domains with well defined tertiary structures, so as to minimize the possibility of affecting the tertiary structure of gp39 ECD while increasing the likelihood of successful expression and transport. For example, an ECD protein which is known to have been incorporated into a fusion protein that was synthesized and exported in high yield from an expression system would be likely to be a suitable tag protein for soluble gp39.

Another criterion for selecting a tag protein is the availability of reagents that react with the tag protein. For example, a tag protein to which one or more monoclonal antibodies have been produced offers the advantage of providing a "handle" which may be detected or manipulated by monoclonal antibody.

Suitable tag proteins include but are not limited to extracellular domains of type I membrane proteins such as CD8, secreted proteins such as IL-4, Fc domains of immunoglobulins, etc. In preferred, specific, nonlimiting embodiments of the invention, the tag protein is the murine CD8 that comprises its extracellular domain (ECD) (described by Nakauchi et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:5126-5130) or its human equivalent (Kavathas et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7688). The nucleotide and amino acid sequences of murine CD8 are presented in Figure 8; the ECD is found between amino acid residues 1 and 174 (numbering from the first ATG of nucleic acid sequence), as encoded by that portion of the nucleic acid between nucleotide residues 121 and 708. The nucleotide and amino acid sequences of corresponding human CD8 are presented in Figure 9; the ECD is found between amino acid residues 1 and 161 as encoded by that portion of the nucleic acid between nucleotide residues 129 and 611.

For example, and not by way of limitation, the construct depicted in Figure 2A and described infra in Section 7 may be used to produce soluble gp39 (sgp39). This construct may be prepared as follows:

The ECD of hgp39 may be amplified from a cDNA library prepared from mRNA from phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes. The oligonucleotide primers may be designed based on the sequence set forth in Figure 1 and may be engineered so as to place a restriction enzyme cleavage site (e.g. a BamHI cleavage site) is at the 5' end of the gene such that the reading frame may be preserved when the chimeric gene is constructed. For example, oligonucleotides which may be used are

5'-CGA

AGC TTG GAT CCG AGG AGG TTG GAC AAG ATA GAA GAT-3' [SEQ. ID NO: 15] and 5'-CGC TCT AGA TGT TCA GAG TTT GAG TAA GCC-3' [SEQ. ID NO: 14].

Polymerase chain reaction may be performed using Pfu polymerase with buffer supplied by the manufacturer (Stratagene, LaJolla, CA) with the following temperature program: 5 min., 95°C; 2 min., 72°C, 2 min., 55°C; 40 cycles of amplification consisting of 1 min., 95°C; 2 min., 55°C; 3 min., 72°C; 10 min., 72°C. The PCR product may be digested with BamHI and XbaI and subcloned into a vector containing the gene encoding either the murine CD8 (Lyt2a) ECD or its human equivalent. The resulting construct may then be transfected into COS cells and then expressed to form sgp39, which may then be purified by absorption and elution from an affinity column which contains either CD40-Ig or an anti-murine CD8 mAb, such as 53-6, immobilized on a solid support such as sepharose beads.

It may be desirable to confirm that sgp39 fusion proteins prepared from the gp39 ECD and various tags are capable of binding to CD40. For example, and not by way of limitation, the binding of sgp39 to CD40 may be confirmed in an ELISA assay in which wells of a 96-well plate may be coated with anti-tag antibody, washed with phosphate buffered saline (PBS) containing 0.05 percent Tween-20 (TPBS) and then blocked with 1X specimen Diluent Concentrate (Genetic Systems, 225 µl/well, 2 hours, room temperature). Wells may then be washed with TPBS. Supernatants from COS cells expressing sgp39 or a negative control may be added (150 µl/well) and plates may be incubated at 4°C overnight. Wells may then be washed with TPBS and then CD40 (e.g. in the form of CD40-Ig fusion protein) or negative control protein, which may desirably be added as serial dilutions in PBS containing 1mM CaCl<sub>2</sub> and 1mM MgCl<sub>2</sub>, 20µg/ml to 0.6µg/ml, 100µl/well, 1 hr., room temp.). Wells may then be washed with TPBS and binding of CD40 to the sgp39-coated wells detected; for example, binding of CD40-Ig to sgp39-coated wells may be detected by adding peroxidase-conjugated goat F(ab')<sub>2</sub> anti-human IgG followed by chromogenic substrate (e.g. Genetic Systems chromogen diluted 1:100 in EIA Buffered Substrate, Genetic Systems, 100µl/well). The chromogenic reaction may be stopped after 10 minutes with the addition of Stop Buffer (Genetic Systems, 100µl/well) and the absorbance may be measured on an ELISA reader at dual wavelengths (450nm, 630nm). Alternatively, ELISA may be performed by immobilization of CD40 (e.g. CD40-Ig) on plates coated with antibody (e.g. goat anti-human Fc), and binding of sgp39 from increasing dilutions of COS cell supernatant may be detected using anti-tag antibody.

Additionally, the ability of sgp39 to bind to CD40 may be ascertained by B cell proliferation assay as follows. Peripheral blood mononuclear cells may be isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). Human B Lymphocytes may be enriched from PBMC by passage of cells over nylon columns (Wako Chemicals USA, Inc., Richmond, VA) and harvesting of adherent cells. The cells may then be treated with leu-leu methyl ester (Sigma, St. Louis, MO) to deplete monocytes and NK cells. The resulting cell population may be analyzed by flow cytometry on an EPICS C (Coulter Electronics, Hialeah, FLA) to determine the percentage of B cells.

Tonsillar B cells may be prepared from intact tonsils by mincing to produce a tonsillar cell suspension. The cells may then be centrifuged through Lymphocyte Separation Medium, washed twice, and then fractionated on a discontinuous Percoll gradient. Cells with a density greater than 50 percent may be collected, washed twice, and used in proliferation assays.

Measurement of proliferation may be performed by culturing B cells in quadruplicate samples in flat-bottomed 96-well microtiter plates at 5 x 10<sup>4</sup> cells per well in complete RPMI medium containing 10 percent fetal calf serum. Supernatants of COS cells expressing sgp39 or control construct, diluted 1:4, plus PMA (10ng/ml, LC Services, Woburn, MA) or 1F5 (anti-CD20, 1µl/ml), may be added to the cultures, and then B-cell proliferation may be measured by uptake of [<sup>3</sup>H]-thymidine (6.7 Ci/mmol; New England Nuclear,

Boston, MA) after 5 days of culture and an overnight pulse (cells may be harvested onto glass fiber filters and radioactivity may be measured in a liquid scintillation counter). A boost in B-cell proliferation above control levels (preferably by at least about 100 percent) associated with a particular form of sgp39 indicates the sgp39 interacts with CD40 on the surface of B cells and is biologically active.

5 The present invention provides for an essentially purified and isolated nucleic acid comprising a sequence substantially as set forth in Figure 1 from nucleotide residues 160 to 787, which may be used toward the production of the fusion proteins of the invention. Accordingly, the present invention also provides for an essentially purified and isolated nucleic acid comprising a sequence substantially as set forth in Figure 1 from nucleotide residues 160 to 787 and further comprising a sequence encoding an  
10 extracellular domain of a protein other than a gp39 protein (that is, human or non-human gp39 protein); in preferred embodiments, this other protein is murine or human CD8 protein. In a specific, nonlimiting embodiment of the invention, the extracellular domain of this other protein is the extracellular domain of murine or human CD8 from amino acid residues 1 to 174 and 1 to 161, respectively as encoded by the sequence between nucleotides 121-708 as depicted in Figure 8 and residues 129-611 in Figure 9. In a  
15 preferred, specific, nonlimiting embodiment of the invention, this essentially purified and isolated nucleic acid is contained in plasmid CDM7B<sup>-</sup> MC1061/p3-shgp39 as deposited with the ATCC and assigned accession number 69049. The present invention further provides for proteins encoded by such nucleic acids.

For example, the present invention provides for an essentially purified and isolated protein comprising a  
20 sequence substantially as set forth in Figure 1 from amino acid residues 47-261, and for this essentially purified and isolated protein further comprising an extracellular domain of a protein other than a gp39 protein. In preferred embodiments, this other protein is murine or human CD8 protein, and in a specific, nonlimiting embodiment of the invention, the extracellular domain of this other protein is the extracellular domain of murine or human CD8 from amino acid residues 1-174 and 1-161, respectively. In a preferred,  
25 specific, nonlimiting embodiment of the invention, the essentially purified and isolated protein is as produced by expression of plasmid CDM7B<sup>-</sup> MC1061/p3-shgp39, as deposited with the ATCC and assigned accession number 69049.

### 5.3. UTILITY OF THE INVENTION

30 The present invention provides for a method of promoting the proliferation and/or differentiation of CD40-bearing cells comprising exposing the cells to an effective concentration of a soluble gp39 protein, such as the soluble gp39 proteins, both human and nonhuman, described in Section 5.2, supra.

In preferred embodiments, the invention is used to promote the proliferation and/or differentiation of B-cells which may have been activated prior to exposure to the soluble gp39 protein, concurrently with  
35 exposure to soluble gp39 protein or, less preferably, after exposure to soluble gp39 protein, wherein the soluble gp39 protein is still present. Activation of B-cells may be accomplished by any method known in the art, including exposure to costimulating agents including, but not limited to, anti-immunoglobulin antibody, antibody directed toward a B-cell surface antigen (e.g. CD20), phorbol myristyl acetate (PMA), ionomycin,  
40 or soluble or surface-bound cytokines (e.g. IL-4).

An effective concentration of soluble gp39 is defined herein as a concentration which results in an increase in activated B-cell proliferation of at least one hundred percent relative to the proliferation of activated B-cells that are not exposed to gp39 or other mediators of B-cell proliferation (see, for example, Section 5.1 supra and Section 7.1.3 infra). For example, and not by way of limitation, a concentration of  
45 between about 0.005-2.5 µg/ml, and most preferably about 0.1-0.25 µg/ml may be used.

As set forth in U. S. Serial No. 708,075, which is incorporated by reference in its entirety herein, the soluble gp39 proteins of the invention have a number of uses, including *in vitro* and *in vivo* uses.

According to one embodiment of the invention, soluble gp39 may be used to produce an *in vitro* cell culture system for long-term B-cell growth. This may be particularly useful in the preparation of antigen-specific B-cell lines.  
50

In another *in vitro* embodiment, soluble gp39 may be used to identify or separate cells which express CD40 antigen and/or to assay body fluids for the presence of the CD40 antigen which may or may not be shed. For example, the binding of soluble gp39 to CD40 antigen may be detected by directly or indirectly labeling the soluble gp39, for example, by incorporating radiolabel or chromogen into the soluble gp39  
55 protein (direct labeling) or via anti-gp39 antibody (indirect labeling). In this manner, soluble gp39 may be used diagnostically *in vitro* to identify CD40 antigen as expressed in tumors, malignant cells, body fluids, etc.



In related embodiments, directly or indirectly labeled soluble gp39 may be used in vivo to image cells or tumors which express the CD40 antigen.

In various other in vivo embodiments, soluble gp39 may be used to increase an immune response, for example, by acting, effectively, as a type of "adjuvant" to increase an immune response to a vaccine. Alternatively, soluble gp39 may be used to increase the immune response of an immunosuppressed individual, such as a person suffering from acquired immunodeficiency syndrome, from malignancy, or an infant or elderly person.

In still further embodiments of the invention, soluble gp39 may be chemically modified so that cells that it binds to are killed. Since all B-cells express CD40, this approach would result in suppression of the immune response. For example, a cytotoxic drug linked to soluble gp39 may be used in vivo to cause immunosuppression in order to cross histocompatibility barriers in transplant patients; alternatively, these modified ligands may be used to control autoimmune diseases.

In further embodiments, soluble gp39 may be used to promote the proliferation and/or differentiation of CD40-bearing cells that are not B cells, for example, sarcoma cells, as a means of directly treating malignancy or as an adjunct to chemotherapy.

The present invention further provides for the production of anti-hgp39 antibodies, polyclonal or monoclonal, using standard laboratory techniques.

The present invention also provides for pharmaceutical compositions that comprise a therapeutically effective concentration of a soluble gp39 as described in Section 5.2, supra, in a suitable pharmacological carrier.

Such pharmaceutical compositions may be administered to a subject in need of such treatment by any suitable mode of administration, including but not limited to intravenous, local injection, subcutaneous, intramuscular, oral, intranasal, rectal, vaginal, intrathecal, etc.

## 25 6. EXAMPLE: THE HUMAN T CELL ANTIGEN GP39, A MEMBER OF THE TUMOR NECROSIS GENE FAMILY, IS A LIGAND FOR THE CD40 RECEPTOR ON B CELLS

### 6.1. MATERIALS AND METHODS

30 CD40-Ig, as described in Noelle et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:6550-6554, was modified by the introduction of three mutations, namely L234F, L235E, and G237A, in the immunoglobulin domain to reduce the binding to Fc receptors. The modified CD40-Ig was purified from COS cell supernatants as previously described (Aruffo *et al.*, 1990, Cell 61:1303-1313). Human gp39 CDNA was amplified by polymerase chain reaction (PCR) from a library prepared from mRNA isolated from PHA-activated human peripheral blood T-cells (Camerini *et al.*, 1989, Nature 342:78-82). The oligonucleotide primers were 35 designed based on the sequence of the murine gp39 (Armitage *et al.*, 1992, Nature, 357:80-82) and included sites for the restriction enzymes Xba I and HindIII to be used in subcloning the PCR product. The oligonucleotides used were:

40 5'-GCG AAG CTT TCA GTC AGC  
ATG ATA GAA ACA-3' [SEQ. ID NO: 13] and 5'-CGC TCT AGA TGT TCA  
GAG TTT GAG TAA GCC-3' [SEQ. ID NO: 14].

45 Amplification was performed with Taq polymerase and the reaction buffer recommended by the manufacturer (Perkin Elmer Cetus Corp., Norwalk, CT) using 30 cycles of the following temperature program: 2 min., 95 °C; 2 min., 55 °C; 3 min., 72 °C. The PCR product was digested with HindIII and XbaI and was found to contain an internal HindIII restriction site. The HindIII-XbaI fragment was subcloned into the CDM8 vector. 50 The complete gene product was constructed by subcloning the HindIII-HindIII fragment into the vector containing the HindIII-XbaI fragment. The resulting construct was transfected into COS cells using DEAE-dextran as described in Aruffo *et al.*, 1990, Cell 61:1303-1313). Transfectants were stained with CD40-Ig (25 µg/ml in DMEM media) followed by FITC-conjugated goat anti-human IgG Fc antibody (1:50 dilution in DMEM, TAGO, Burlingame, CA) and visualized by immunofluorescence microscopy. The complete human 55 gp39 was obtained by colony hybridization as described (Sambrook et al., 1989, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The subcloned HindIII-HindIII fragment of the PCR product was used to generate a <sup>32</sup>p-labeled probe by random primed polymerization. Plasmid DNA from three individual clones were transfected into COS cells and cells were

stained with CD40-Ig. One clone, clone 19, was positive by this criteria and was used in the remainder of the study. The sequence was determined by dideoxy sequencing using Sequenase™ (United States Biochemical Co., Cleveland, OH)

## 6.2. RESULTS

A cDNA encoding the human gp39 was amplified from a cDNA library prepared from mRNA isolated from PHA activated human peripheral blood T cells by the polymerase chain reaction (PCR) using synthetic oligonucleotides based on the murine gp39 sequence (Armitage *et al.*, 1992, Nature 357:80-82). The PCR product was subcloned into the expression vector CDM8 (Seed, 1987, Nature 329:840-842). COS cells transfected with the CDM8-gp39 plasmid produced protein which bound to CD40-Ig (Noelle *et al.*, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:6550-6554). A complete human gp39 gene was isolated by colony hybridization from the same cDNA library that was used for the PCR amplification of gp39 using the subcloned PCR product as a probe. A number of positive clones were isolated and analyzed by restriction enzyme digestion. DNA corresponding to those clones containing the largest inserts, 1.8-1.5 kb, were transfected into COS cells and their ability to direct the expression of a CD40-Ig binding protein examined. One such clone was positive by this criteria and was analyzed further and is referred to hereafter as human gp39. Immunoprecipitation of cDNA-encoded human gp39 protein from transfected COS cells using CD40-Ig showed a single band corresponding to a molecular mass of about 32-33 kDa. The COS-cell derived protein is smaller than we had expected based on our previous studies of murine gp39, however, we have observed in many instances that the apparent molecular masses of a number of different T cell surface proteins obtained from COS cell transfectants are smaller than those obtained from T cells (Aruffo and Seed, 1987, EMBO J. 11:3313-3316; Aruffo *et al.*, 1991, J. Exp. Med. 174:949-952). These differences in size may be the result of incomplete glycosylation of the proteins by COS cells.

The human gp39 cDNA is about 1.8 kb in length and encodes a polypeptide of 261 amino acids (aa) with a predicted molecular mass of about 29 kDa consisting of a 22 amino acid amino-terminal cytoplasmic domain, a 24 amino acid hydrophobic transmembrane domain and a 215 amino acid carboxyterminal extracellular (EC) domain with one N-linked glycosylation site (Asn-X-Ser/Thr) in the EC and one in the cytoplasmic domain (nucleotide sequences corresponding to coding sequence and the predicted amino acid sequence are shown in Figure 1a). The expected orientation of the protein, with an extracellular carboxy-terminus, classifies it as a type II membrane protein and the difference between the predicted and observed molecular mass suggest that it undergoes posttranslational modifications, most likely the addition of carbohydrate groups.

The predicted amino acid sequence of human gp39 was compared with those in the National Biomedical Research Foundation (NBRF) database using the FASTP algorithm and found to have significant homology with tumor necrosis factor (TNF)  $\alpha$  (Gray *et al.*, 1984, Nature 312:721-724) and  $\beta$  (Pennica *et al.*, 1984, Nature 312:724-729; Wang *et al.*, 1985, Science 228:149-154) (Figure 1b). The extracellular domain of gp39 is as closely related to TNF  $\alpha$  and  $\beta$ , having about 25% homology with each, just as TNF  $\alpha$  and TNF  $\beta$  share about 30% homology (Pennica *et al.*, 1984, Nature 312:724-729).

## 6.3. DISCUSSION

The ability of the surface receptor CD40 to deliver signals to the B cell has been established using monoclonal antibodies (Clark and Ledbetter, 1986, Proc. Natl. Acad. Sci. 83:4494-4498; Gordon *et al.*, 1987, Eur. J. Immunol. 17:1535-1538). To further study the role of CD40, a cDNA encoding the CD40 ligand from a human source has been isolated and characterized.

Isolation of a cDNA clone encoding human gp39 showed that this type II membrane protein is closely related to TNF  $\alpha$  (Gray *et al.*, 1984, Nature 312:721-724) and  $\beta$  (Pennica *et al.*, 1984, Nature 312:724-729; Wang *et al.*, 1985, Science 228:149-154). TNF  $\alpha$  and  $\beta$  are pleiotropic cytokines that exist predominantly as secreted proteins.

## 7. EXAMPLE: EXPRESSION OF A SOLUBLE FORM OF GP39 WITH B CELL CO-STIMULATORY ACTIVITY

### 7.1. MATERIALS AND METHODS

#### 5 7.1.1. CONSTRUCTION, CHARACTERIZATION, AND PREPARATION OF A SOLUBLE GP39 CHIMERA

The extracellular domain of the human gp39 was amplified from the cDNA library prepared from mRNA from PHA activated human peripheral blood lymphocytes. The oligonucleotide primers were designed based on sequence information obtained from the PCR product described above and were designed to place a BamHI site at the 5' end of the gene such that the reading frame would be preserved when the chimeric gene was constructed. The oligonucleotides used were

5' - CGA

15 AGC TTG GAT CCG AGG AGG TTG GAC AAG ATA GAA GAT-3' [SEQ. ID NO: 15] and 5'-CGC TCT AGA TGT TCA GAG TTT GAG TAA GCC-3' [SEQ. ID NO : 14].

20 The PCR was performed using the Pfu polymerase with the buffer supplied by the manufacturer (Stratagene, La Jolla, CA) with the following temperature program: 5 minutes, 95 °C; 2 minutes, 72 °C; 2 minutes, 55 °C; 40 cycles of amplification consisting of 1 minute, 95 °C; 2 minutes, 55 °C; 3 minutes, 72 °C; 10 minutes, 72 °C. The PCR product was digested with BamHI and XbaI and subcloned in a vector containing the gene encoding the murine CD8 (Lyt2a) extracellular domain with a BamHI restriction site generated by PCR. Similarly, the gene encoding the extracellular domain of human CD72 was generated by PCR to contain a BamHI restriction site and subcloned in the CD8-containing vector in the same manner.

The ability of COS cells to express and export shgp39 and sCD72 was tested. First, COS cells were transfected using DEAE-dextran. One day after transfection, cells were trypsinized and replated. One day later, cells were fixed with 2% formaldehyde in PBS (20 min., room temp.) and permeabilized with 2% formaldehyde in PBS containing 0.1% Triton X-100. (20 min., room temp.). Cells transfected with sgp39 were stained with CD40-Ig (25 µg/ml in DMEM, 30 min., room temp.) followed by FITC-conjugated goat anti-human Fc antibody (TAGO, Burlingame, CA) diluted 1:500 in DMEM. Cells transfected with sCD72 were stained with the anti-CD72 antibody BU40 (The Binding Site, Birmingham, UK) followed by FITC-conjugated goat anti-mouse Fc antibody (TAGO, Burlingame, CA) diluted 1:500 in DMEM.

COS cells transfected with the shgp39 or sCD72 constructs or vector alone (mock) were grown overnight in Cys- and Met- free DMEM to which [<sup>35</sup>S]-L-methionine and [<sup>35</sup>S]-L-cysteine had been added (Tran[<sup>35</sup>S]-label, ICN, Costa Mesa, CA, 27 µCi/ml). Supernatants were harvested and centrifuged at 1krpm for 10 minutes. Fusion proteins were recovered from the supernatant using CD40-Ig, 53-6 (anti-murine CD8) plus goat anti-rat Fc, BU40, BU41 (The Binding Site, Birmingham, UK) plus goat anti-mouse IgM Fc, or J3.101 (AMAC Inc., Westbrook, ME). Goat antibodies were purchased from Organon Teknika Co., West Chester, PA. For each sample, 1 ml of supernatant, 75 µl Protein A-sepharose (Repligen, Cambridge, MA) and the precipitating agent(s) were mixed and incubated at 40 °C for 2 hr. The sepharose was washed extensively with PBS containing 0.01% NP-40 and resuspended in loading buffer containing 5% β-mercaptoethanol. Proteins were subjected to SDS-PAGE in a 8% polyacrylamide gel. The gel was fixed, dried and exposed to film. COS cell supernatants containing shgp39 or sCD72 were generated by transfection of COS cells. One day after transfection, cell media was changed to DMEM containing 2% FBS. Supernatants were harvested eight days after transfection.

#### 50 7.1.2. BINDING ASSAYS

The binding of hgp39 and CD40 to the soluble forms of their respective ligands was tested by staining of transfected COS cells. COS cells were transfected with CD40, hgp39 or vector alone (mock) using DEAE-dextran. One day after transfection, cells were trypsinized and replated. Cells were stained on the following day. Cells expressing gp39 or mock transfected cells were stained with CD40-Ig (25 µg/ml) followed by FITC-conjugated goat anti-human Fc. Cells expressing CD40 were stained by incubation with COS cell supernatants containing shgp39 followed by mAb 53-6 (anti-murine CD8, 2.5 µg/ml) then FITC-conjugated goat anti-rat Fc (Organon Teknika Co., West Chester, PA, 1.5 µg/ml). As controls, COS cells expressing

CD40 were stained with FITC-conjugated G28-5 (anti-CD40) or using COS cell supernatants containing sCD72. All incubations were done at room temperature in PBS containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 2% FBS and the same buffer was used for all washes. Following staining, cells were fixed with 1% paraformaldehyde in PBS.

5 The binding of shgp39 to CD40-Ig was investigated in an ELISA assay. Wells of a 96-well plate (Immunolon-2, Dynatech) were coated with 53-6 antibody (anti-murine CD8, 10 µg/ml, 100 µl/well, 50 mM sodium bicarbonate, pH 9.6, 1 hour, room temperature). Wells were washed with phosphate buffered saline containing 0.05% Tween-20 (TPBS) and blocked with 1X Specimen Diluent Concentrate (Genetic Systems, 225 µl/well, 2 hours, room temperature). Wells were washed (TPBS). Supernatants from COS cells  
10 expressing either sgp39 or sCD72 were added (150 µl/well) and plates were incubated at 4°C overnight. Wells were washed (TPBS) and fusion proteins CD40-Ig or Leu8-Ig were added (serially diluted in PBS containing 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, 20 µg/ml to 0.6 µg/ml, 100 µl/well, 1 hr., room temp.) Wells were washed (TPBS) and peroxidase-conjugated goat F(ab')<sub>2</sub> anti-human IgG was added to each well (TAGO, Burlingame CA, 1:5000 dilution in 1X Specimen Diluent, 100 µl/well, 1 hr., room temp.) Wells were washed  
15 (TPBS) and chromogenic substrate was added (Genetic Systems chromogen diluted 1:100 in EIA Buffered Substrate, Genetic Systems, 100 µl/well). The reaction was stopped after 10 minutes with the addition of Stop Buffer (Genetic Systems, 100 µl/well) and the absorbance was measured on an ELISA reader at dual wavelengths, namely 450 or 630nm. Additionally, the ELISA was performed by immobilization of CD40Ig on plates coated with goat anti-human Fc. Binding of shgp39 from increasing dilutions of COS cell super-  
20 natants was detected using 53-6 Mab followed by FITC conjugated goat anti-rat Fc. Fluorescence was measured on a microplate reader.

### 7.1.3. B CELL PROLIFERATION ASSAYS

25 Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). Human B lymphocytes were enriched from PBMC by passage of cells over nylon columns (Wako Chemicals USA, Inc., Richmond, VA) and harvesting of adherent cells. These cells were then treated with leu-leu methyl ester (Sigma, St. Louis, MO) to deplete monocytes and NK cells. The resulting cell population was analyzed by flow cytometry on an EPICS C  
30 (Coulter Electronics, Hialeah, FLA) and consisted of 50% human peripheral B cells.

Tonsillar B cells were prepared from intact tonsils by mincing to give a tonsillar cell suspension. The cells were then centrifuged through Lymphocyte Separation Medium, washed twice and fractionated on a discontinuous Percoll (Sigma, St. Louis, MO) gradient. Cells with a density greater than 50% were collected, washed twice and used in proliferation assays.

35 COS cells transfected with the gp39 construct or vector alone (mock-COS) were harvested from tissue culture plates with EDTA, washed twice with PBS, suspended at  $5 \times 10^6$  cells/ml and irradiated with 5000 rads from a 137 Cs source. COS cells were used at a ratio of 1:4 ( $1 \times 10^4$  COS cells vs.  $4 \times 10^4$  B cells) in proliferation assays.

40 Measurement of proliferation was performed by culturing cells in quadruplicate samples in flat-bottomed 96-well microtiter plates at  $5 \times 10^4$  cells per well in complete RPMI medium containing 10% FCS. Reagents used were IF5 (anti-CD20, 1 µg/ml); PMA (10 ng/ml, LC Services Woburn, MA); G28-5 (anti-CD40, 1 µg/ml); CD40Ig (5 µg/ml in assays of peripheral blood B cells, 20 µg/ml in assays of tonsillar B cells); supernatants of COS cells expressing shgp39 or sCD72 (diluted 1:4). Cell proliferation was measured by uptake of [<sup>3</sup>H]-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) after 5 days of culture and an overnight pulse.  
45 Cells were harvested onto glass fiber filters and radioactivity was measured in a liquid scintillation counter.

## 7.2. RESULTS

### 7.2.1. PREPARATION AND CHARACTERIZATION OF THE RECOMBINANT GP39 AS A CHIMERIC FUSION PROTEIN

50 Because gp39 is a type II membrane protein, and type II membrane proteins are oriented with a carboxy-terminal EC domain, a fusion construct was designed such that a tag polypeptide was placed amino-terminal to the EC portion of the protein, replacing the trans-membrane and cytoplasmic domains of  
55 the surface protein. The tag polypeptide should contain an amino-terminal secretory signal sequence to allow export of the fusion protein. We chose the murine CD8 EC domain (Nakauchi *et al.*, 1985, Proc. Natl. Acad. Sci. U.S.A. 82:5126-5130) as our tag polypeptide to construct our fusion proteins of type II membrane proteins for four reasons: (i) the use of an intact extracellular protein domain with a well defined tertiary

structure as the tag polypeptide minimizes the chances that the tag polypeptide will affect the tertiary structure of the surface protein to which it is fused while maximizing the likelihood that the fusion protein will be expressed and exported, (ii) a previously studied CD8 Ig chimera demonstrated that CD8 fusion proteins are produced and exported by COS cells in high yield, (iii) a large number of mAb directed to CD8 are available and can be used to manipulate the recombinant CD8 fusion proteins; and (iv) the interaction between murine CD8 and human MHC I is not detectable. To generate the CD8-gp39 fusion gene, shgp39, a cDNA fragment encoding the EC domain of murine CD8 was fused with a cDNA fragment encoding the EC domain of gp39 as described in the Materials and Methods (Figure 2a). The shgp39 protein was prepared by transient expression in COS cells and recovered from COS cell supernatants with anti-CD8 mAb or with a soluble recombinant CD40-Ig chimera which we used in our earlier murine gp39 studies (Figure 2b). The shgp39 protein has a molecular mass of about 50 kDa (Figure 2b) when analyzed by SDS-PAGE under reducing conditions. Experimental results indicate that shgp39 forms dimers and trimers in solution.

As a control, a chimeric gene encoding a soluble recombinant form of the B cell antigen CD72 (Von Hoegen *et al.*, 1990, J. Immunol. 144:4870-4877), another type II membrane protein, was constructed (Figure 2a). The sCD72 protein was also produced by transient expression in COS cells and recovered from COS cell supernatants with anti-CD8 mAb or with three anti-CD72 mAb tested, but not with the CD40-Ig fusion protein (Figure 2c).

To further characterize the interaction between CD40 and the soluble recombinant hgp39, COS cells were transfected with a cDNA encoding the full length CD40 protein (Stamenkovic *et al.* 1989, EMBO J. 8:1403-1410) and their ability to bind to shgp39, sCD72, and anti-CD40 mAb examined by fluorescence microscopy. Both the shgp39 and the anti-CD40 mAb bound to the transfectants while sCD72 did not (Figure 3). In addition, COS cells were transfected with a cDNA encoding the surface bound gp39 and their ability to bind to CD40-Ig (Noelle *et al.*, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:6550-6554) or an irrelevant Ig fusion protein, Leu8-Ig (Aruffo *et al.* 1992, Proc. Natl. Acad. Sci. U.S.A. 89:2292-2296), examined. CD40-Ig, but not Leu8-Ig, bound to gp39 expressing COS cells (Figure 3). In parallel experiments, shgp39 and CD72 were immobilized in the wells of a 96 well microtiter dish via an anti-CD8 mAb and their binding to increasing concentrations of CD40-Ig or a control immunoglobulin fusion protein, Leu8-Ig; examined. The binding of CD40-Ig to immobilized shgp39 was saturable, while CD40-Ig did not bind to sCD72 and Leu8-Ig did not bind to shgp39 (Figure 4).

#### 7.2.2. HUMAN GP39 REQUIRES A CO-STIMULUS TO INDUCE B CELL PROLIFERATION

To examine the role of gp39-CD40 interactions in B cell activation, COS cells transfected with either the cDNA encoding hgp39 or vector alone (mock) were tested for their ability to stimulate B cell proliferation. Resting, peripheral blood B cells proliferated only weakly when incubated with hgp39-expressing COS cells alone (Figure 5). However, upon exposure to hgp39-expressing COS cells in conjunction with either (i) IF5 mAb (Clark *et al.*, 1985, Proc. Natl. Acad. Sci. U.S.A. 82:1766-1770), directed against the B cell surface protein CD20, or (ii) PMA, vigorous B cell proliferation was observed. In both cases, the hgp39-driven B cell proliferation could be reduced to background levels with the soluble CD40-Ig fusion protein (Figure 5). B cells proliferated weakly when incubated with mock transfected COS cells in the presence of either the anti-CD20 mAb or PMA and this proliferation was unaffected by the presence of CD40-Ig (Figure 5). The weak B cell proliferation observed with hgp39-expressing COS cells in the absence of a co-stimulatory signal suggests that in this case COS cells also provide co-stimulatory signals that synergize with CD40 signals to drive B cell proliferation.

Resting, human peripheral blood B cells were incubated with the soluble recombinant hgp39, shgp39, or a control soluble fusion protein, sCD72, in the absence or presence of anti-CD20 mAb or PMA. Although very weak proliferation was observed with shgp39 alone, shgp39 induced vigorous B cell proliferation when either anti-CD20 mAb or PMA was present (Figure 6). B cell proliferation was not observed with sCD72, anti-CD20 mAb or PMA alone or with sCD72 in conjunction with anti-CD20 mAb or PMA (Figure 6).

In parallel experiments resting, dense human tonsillar B cells were prepared as described in the Materials and Methods section and their ability to proliferate in response to shgp39 and sCD72 examined (Figure 7). As had been seen with peripheral blood B cells, tonsillar B cells proliferated weakly in response to shgp39 but showed strong proliferation when incubated with shgp39 in the presence of the anti-CD20 mAb IF5 or PMA. No significant proliferation over background levels was observed when the cells were incubated with sCD72 alone or in the presence of the IF5 mAb or PMA. To examine the specificity of the shgp39 driven activation response the ability of CD40-Ig to block the shgp39/IF5 or shgp39/PMA driven B cell proliferation was examined. CD40-Ig was able to reduce the shgp39 driven B cell activation (~20 µg/ml

gave ~50% inhibition, Figure 7A) while a control fusion protein Leu-8-Ig had no effect (Figure 7B).

### 7.3. DISCUSSION

It has been reported that purified murine splenic B cells and human tonsillar B cells proliferate when incubated with CV1/EBNA cells expressing murine gp39 in the absence of co-stimulus (Armitage *et al.*, 1992, Nature 357:80-82). Based on these data it had been thought that gp39 is directly mitogenic for B cells. To determine if gp39 binding to CD40 is able to stimulate resting B cells to proliferate in the absence of other co-stimulatory signals, and the effect of the fibroblast cells in the stimulation, the proliferation of B cells in response to COS cells expressing full length hgp39 or shgp39 was tested. In contrast to the teachings of Armitage, *supra*, which suggest that gp39 must be associated with a membrane to be active, our results show that the hgp39 was active in both membrane-associated and soluble forms; however, interesting differences between hgp39<sup>+</sup> COS cells and shgp39 were seen. COS cells expressing hgp39 were able to induce only weak B cell proliferation in the absence of co-stimuli but could synergize with co-stimuli such as anti-CD20 mAb or PMA to induce vigorous B cell proliferation. In all cases, the B cell proliferation could be reduced to background levels with soluble recombinant hgp39 receptor, CD40-Ig.

shgp39 was only able to induce resting B cells, isolated from either peripheral blood or tonsils, to proliferate in conjunction with co-stimuli such as anti-CD20 mAb or PMA. As had been observed with hgp39-expressing COS cells, shgp39 driven B cell activation could be inhibited with CD40-Ig but not with an irrelevant Ig fusion protein.

These data indicate that hgp39 requires a co-stimulatory signal to most effectively drive B cell proliferation and that there is no strict requirement for cell surface expression of hgp39 for activity. In addition, the ability of hgp39 expressed on the surface of COS cells to stimulate weak B cell proliferation supports the idea that COS cells may also provide low level co-stimulatory signals, as yet undefined, that can synergize with those provided by hgp39.

The development of factor dependent, long term B cell cultures has important applications for the study of B cell growth and differentiation and the development of antigen-specific B cell lines (Tisch *et al.*, 1988, Immunol. Today 9:145-150). Experiments with anti-CD40 mAb showed that CD40 signals can synergize with other co-stimulatory signals such as those delivered by anti-CD20 mAb to drive B cell proliferation and that treatment of B cells with anti-CD40 mAb induces a state of B cell "alertness" which allows them to respond more readily to subsequent activation signals. The ability of shgp39 to stimulate B cell proliferation in conjunction with anti-CD20 mAb or PMA suggests that it may be used to create *in vitro* systems for long term B cell growth.

It is interesting to note that the CD40-Ig fusion protein and the shgp39 fusion described here can be used to, respectively, either inhibit or stimulate the CD40 response in B cells and thus are useful tools in the study of B-cell/T cell interactions and in clinical applications.

### 8. DEPOSIT OF MICROORGANISMS

The following were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852:

	ATCC Designation
<u>Escherichia coli</u> CDM7B <sup>-</sup> MC1061/p3-shgp39	69049
<u>Escherichia coli</u> CDM8 MC1061/p3-hgp39	69050

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiments are intended as illustrations of single aspects of the invention and any microorganisms which are functionally equivalent are within the scope of the invention.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

Various publications have been cited herein, the contents of which are hereby incorporated by reference in their entirety.

## SEQUENCE LISTING

5

## (1) GENERAL INFORMATION

(i) APPLICANT:

(A) NAME: Bristol-Myers Squibb Company  
(B) STREET: 345 Park Avenue  
(C) CITY: New York  
(D) STATE: New York  
(E) COUNTRY: U.S.A.  
(F) ZIP: 10154

## (ii) TITLE OF INVENTION: SOLUBLE LIGANDS FOR CD40

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Reitstötter, Kinzebach & Partner  
(B) STREET: Sternwartstraße 4  
(C) CITY: Munich  
(D) STATE: Bavaria  
(E) COUNTRY: Germany  
(F) ZIP: D-81679

(v) **COMPUTER READABLE FORM:**

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release 1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE: September 03, 1993  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kinzebach, Werner, Dr.  
(B) REGISTRATION NUMBER: 3379  
(C) REFERENCE/DOCKET NUMBER: M/34164

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (089) 98 06 56  
(B) TELEFAX: (089) 98 73 04  
(C) TELEX: 5215208

## (2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 840 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (11) MOLECULE TYPE: DNA (genomic)

(1x) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 22..807

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCATTTCAAC	TTTAAACACAG	C	ATG	ATC	GAA	ACA	TAC	AAC	CAA	ACT	TCT	CCC	51			
		Met	Ile	Glu	Thr	Tyr	Asn	Gln	Thr	Ser	Pro	10				
CGA	TCT	GCG	GCC	ACT	GGA	CTG	CCC	ATC	AGC	ATG	AAA	ATT	TTT	ATG	TAT	99
Arg	Ser	Ala	Ala	Thr	Gly	Leu	Pro	Ile	Ser	Met	Lys	Ile	Phe	Met	Tyr	
				15					20					25		
TTA	CTT	ACT	GTT	TTT	CTT	ATC	ACC	CAG	ATG	ATT	GGG	TCA	GCA	CTT	TTT	147
Leu	Leu	Thr	Val	Phe	Leu	Ile	Thr	Gln	Met	Ile	Gly	Ser	Ala	Leu	Phe	

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	30	35	40	
5	GCT GTG TAT CTT CAT AGA AGG Ala Val Tyr 45	TTG CAC AAG ATA GAA GAT Leu Asp Lys 50	GAT GAA AGG AAT Asp Glu Arg Asn 55	195
	CTT CAT GAA GAT TTT GTA TTC Leu His Glu Asp Phe Val 60	ATG AAA ACG ATA CAG AGA Met Lys Thr Ile Gln Arg 65	TGC AAC ACA Cys Asn Thr 70	243
10	GGA GAA AGA TCC TTA TCC Gly Glu Arg Ser Leu 75	CTG AAC TGT GAG GAG Leu Asn Cys Glu Glu 80	ATT AAA AGC CAG Ile Lys Ser Gln 85	291
	TTT GAA GGC TTT GTG AAG GAT Phe Glu Gly Phe 95	ATA ATG TTA AAC AAA Ile Met Leu Asn Lys 100	GAG GAG ACG AAG Glu Glu Thr Lys 105	339
15	AAA GAA AAC AGC TTT GAA ATG Lys Glu Asn Ser Phe 110	CAA AAA GGT GAT CAG AAT Gln Lys Gly Asp Gln Asn 115	CCT CAA ATT Pro Gln Ile 120	387
	GCG GCA CAT GTC ATA AGT GAG Ala Ala His Val Ile 125	GCC AGC AGT AAA ACA Ala Ser Ser Lys Thr 130	ACA TCT GTG TTA Ser Val Leu 135	435
20	CAG TGG GCT GAA AAA GGA Gln Trp Ala Glu Lys 140	TAC TAC ACC ATG AGC AAC Tyr Tyr Thr Met Ser 145	AAC AAC TTG GTA ACC Asn Asn Leu Val Thr 150	483
	CTG GAA AAT GGG AAA CAG Leu Glu Asn Gly Lys 155	CTG ACC GTT AAA AGA Leu Thr Val Lys Arg 160	CAA GGA CTC TAT TAT Gln Gly Leu Tyr Tyr 165	531
25	ATC TAT GCC CAA GTC ACC TTC Ile Tyr Ala Gln Val 175	TGT TCC AAT CGG GAA GCT Cys Ser Asn Arg Glu Ala 180	TCG AGT CAA Ser Ser Gln 185	579
	GCT CCA TTT ATA GCC AGC CTC Ala Pro Phe Ile Ala 190	CTC TGC CTA AAG TCC CCC Leu Cys Leu Lys Ser Pro 195	GGT AGA TTC GAG Gly Arg Phe Glu 200	627
30	AGA ATC TTA CTC AGA GCT GCA Arg Ile Leu Leu Arg 205	AAT ACC CAC AGT TCC GCC Asn Thr His Ser Ser 210	AAA CCT TGC Lys Pro Cys 215	675
35	GGG CAA CAA TCC ATT CAC Gly Gln Gln Ser Ile 220	TTG GGA GGA GTA TTT GAA Leu Gly Gly Val Phe 225	TTG CAA CCA GGT Glu Leu Gln Pro Gly 230	723
	GCT TCG GTG TTT GTC AAT GTG Ala Ser Val Phe Val 235	ACT GAT CCA AGC CAA GTG Thr Asp Pro Ser Gln Val 240	AGC CAT GGC Ser His Gly 245	771
40	ACT GGC TTC ACG TCC TTT GGC Thr Gly Phe Thr Ser 255	TTA CTC AAA CTC Leu Leu Lys Leu 260	TGAACAGTGT CACCTGACG	824
45	GCTGTGGTGG AGCTGA			840

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 261 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ile Glu Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly  
1 5 10 15  
Leu Pro Ile Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu  
20 25 30  
Ile Thr Gln Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg  
35 40 45  
Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val  
50 55 60  
Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser  
65 70 75 80  
Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys  
85 90 95  
Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu  
100 105 110  
Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser  
115 120 125  
Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly  
130 135 140  
Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln  
145 150 155 160  
Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr  
165 170 175  
Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser  
180 185 190  
Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala  
195 200 205  
Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His  
210 215 220  
Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn  
225 230 235 240  
Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe  
245 250 255  
Gly Leu Leu Lys Leu  
260

## (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 151 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val  
1 5 10 15

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Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu  
 20 25 30  
 Lys Gly Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly  
 35 40 45  
 Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln  
 50 55 60  
 Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile  
 65 70 75 80  
 Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu  
 85 90 95  
 Arg Ala Ala Asn Thr His Ser Ser Ala Lys Leu Gly Gly Gln Gln Ser  
 100 105 110  
 Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe  
 115 120 125  
 Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr  
 130 135 140  
 Ser Phe Gly Leu Leu Lys Leu  
 145 150

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 151 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Glu Met Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val  
 1 5 10 15  
 Val Ser Glu Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys  
 20 25 30  
 Lys Gly Tyr Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly  
 35 40 45  
 Lys Gln Leu Thr Val Lys Arg Glu Gly Leu Tyr Tyr Tyr Tyr Thr Gln  
 50 55 60  
 Val Thr Phe Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile  
 65 70 75 80  
 Val Gly Leu Trp Leu Lys Pro Ser Ile Gly Ser Glu Arg Ile Leu Leu  
 85 90 95  
 Lys Ala Ala Asn Thr His Ser Ser Ser Gln Leu Cys Glu Gln Gln Ser  
 100 105 110  
 Val His Leu Gly Gly Tyr Phe Glu Leu Gln Ala Gly Ala Ser Val Phe  
 115 120 125  
 Val Asn Val Thr Glu Ala Ser Gln Tyr Ile His Arg Val Gly Phe Ser  
 130 135 140  
 Ser Phe Gly Leu Leu Lys Leu  
 145 150

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## (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 157 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1          5          10          15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20          25          30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
35          40          45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
50          55          60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65          70          75          80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85          90          95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
100         105         110

Pro Trp Tyr Glu Pro Ile Tyr Ile Gly Gly Val Phe Gln Leu Glu Lys
115         120         125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
130         135         140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145         150         155
  
```

## (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 155 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Pro Lys Met His Leu Ala His Ser Thr Leu Lys Pro Ala Ala His Leu
1          5          10          15

Ile Asn Asp Pro Ser Lys Gln Asn Ser Leu Leu Trp Arg Ala Asn Thr
20          25          30

Asp Arg Ala Phe Leu Gln Asp Gly Phe Ser Leu Ser Ser Asn Asn Ser
35          40          45

Leu Leu Val Pro Thr Ser Gly Ile Tyr Phe Tyr Tyr Ser Gln Val Val
50          55          60

Phe Ser Gly Lys Ala Tyr Ser Pro Lys Ala Thr Ser Ser Pro Ile Tyr
65          70          75          80
  
```

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Leu Ala His Glu Val Gln Leu Phe Ser Ser Gln Tyr Pro Phe His Val  
85 90 95  
Pro Leu Leu Ser Ser Gln Lys Met Val Tyr Pro Gly Leu Gln Glu Pro  
100 105 110  
5 Trp Leu His Ser Met Tyr His Gly Ala Ala Phe Gln Leu Thr Gln Gly  
115 120 125  
Asp Gln Leu Ser Thr His Thr Asp Gly Ile Pro His Leu Val Leu Ser  
130 135 140  
10 Pro Ser Thr Val Phe Phe Gly Ala Gly Ala Leu  
145 150 155

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear  
15  
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Asp Phe Ala Cys Asp Pro Asp Pro Arg Arg Leu Asp Lys Ile  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear  
25  
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Asp Phe Ala Cys Asp Pro Asp Pro Arg Tyr Leu Gln Val Ser  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 972 base pairs  
(B) TYPE: nucleic acid  
(D) TOPOLOGY: linear  
35  
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTGGCTAAA GGAGCAGTTT CCCCGACCCT ACACGCCTCC CCCACCGCAC CTCCTCCGCC 60  
45 CTGTTCTCTGG GCCCTCCTCC TAGAGCCCTA GCTTGACCTA AGCTGCTTGC TGGTGGAGAG 120  
CACACCATGG CCTACCGTT GACCCGCTTT CTGTCGCTGA ACCTGCTGCT GCTGGGTGAG 180  
TCGATTATCC TGGGGACTGG AGAAGCTAAG CCACAGGCAC CCGAACTCCG AATCTTTCCA 240  
50 AAGAAAATGG ACGCCGAAC TGGTCAGAAG GTGGACCTGG TATGTGAAGT GTTGGGGTCC 300

55

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GTTTCGCAAG GATGCTCTTG GCTCTTCCAG AACTCCAGCT CCAAACCTCC CCAGCCCACC 360  
 TTCGTTGTCT ATATGGCTTC ATCCACAAC AAGATAACGT GGGACGAGAA GCTGAATTCG 420  
 TCGAAACTGT TTTCTGCCAT GAGGGACACG AATAATAAGT ACGTTCTCAC CCTGAACAAG 480  
 5 TTCAGCAAGG AAAACCAAGG CTAATTTTC TGCTCAGTCA TCAGCAACTC GGTGATGTAC 540  
 TTCAGTTCTG TCGTGCCAGT CCTTCAGAAA GTGAACCTCA CTAATACCAA GCCAGTGCTG 600  
 CGAACTCCCT CACCTGTGCA CCCTACCGGG ACATCTCAGC CCCAGAGACC AGAAGATTGT 660  
 10 CGGCCCCGTG GCTCAGTGAA GGGGACCGGA TTGGACTTCG CCTGTGATAT TTACATCTGG 720  
 GCACCCTTGG CCGGAATCTG CGTGGCCCTT CTGCTGTCTT TGATCATCAC TCTCATCTGC 780  
 TACCACAGGA GCCGAAAGCG TGTTCGAAA TGTCCAGGC CGCTAGTCAG ACAGGAAGGC 840  
 AAGCCCAGAC CTTCAGAGAA AATTGTGTAA AATGGCACCG CCAGGAAGCT ACAACTACTA 900  
 15 CATGACTTCA GAGATCTCTT CTTGCAAGAG GCCAGGCCCT CTTTTTCAA GTTTCCTGCT 960  
 GTCTTATGTA TT 972

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 249 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
 Met Ala Ser Pro Leu Thr Arg Phe Leu Ser Leu Asn Leu Leu Leu Leu  
 1 5 10 15  
 Gly Glu Ser Ile Ile Leu Gly Ser Gly Glu Ala Lys Pro Gln Ala Pro  
 20 25 30  
 Glu Leu Arg Ile Phe Pro Lys Lys Met Asp Ala Glu Leu Gly Gln Lys  
 35 40 45  
 Val Asp Leu Val Cys Glu Val Leu Gly Ser Val Ser Gln Gly Cys Ser  
 50 55 60  
 35 Trp Leu Phe Gln Asn Ser Ser Ser Lys Leu Pro Gln Pro Thr Phe Val  
 65 70 75 80  
 Val Tyr Met Ala Ser Ser His Asn Lys Ile Thr Trp Asp Glu Lys Leu  
 85 90 95  
 40 Asn Ser Ser Lys Leu Phe Ser Ala Met Arg Asp Thr Asn Asn Lys Tyr  
 100 105 110  
 Val Leu Thr Leu Asn Lys Phe Ser Lys Glu Asn Glu Gly Tyr Tyr Phe  
 115 120 125  
 Cys Ser Val Ile Ser Asn Ser Val Met Tyr Phe Ser Ser Val Val Pro  
 130 135 140  
 45 Val Leu Gln Lys Val Asn Ser Thr Thr Thr Lys Pro Val Leu Arg Thr  
 145 150 155 160  
 Pro Ser Pro Val His Pro Thr Gly Thr Ser Gln Pro Gln Arg Pro Glu  
 165 170 175

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Asp Cys Arg Pro Arg Gly Ser Val Lys Gly Thr Gly Leu Asp Phe Ala  
 180 185 190  
 Cys Asp Ile Tyr Ile Trp Ala Pro Leu Ala Gly Ile Cys Val Ala Leu  
 195 200 205  
 Leu Leu Ser Leu Ile Ile Thr Leu Ile Cys Tyr His Arg Ser Arg Lys  
 210 215 220  
 Arg Val Cys Lys Cys Pro Arg Pro Leu Val Arg Gln Glu Gly Lys Pro  
 225 230 235 240  
 Arg Pro Ser Glu Lys Ile Val Asn Gly  
 245

## (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1060 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGCTCCCGC GCGCCTCCCC CTCGCGCCCG AGCTTCGAGC CAAGCAGCGT CCTGGGGAGC 60  
 GCGTCATGGC CTTACCACTG ACCGCTTGC TCCTGCGGCT GGCCTTGCTG CTCCACGCCG 120  
 CCAGGCCGAG CCAGTTCCGG GTGTGCGCGC TGGATCGGAC CTGGAACCTG GCGAGACAG 180  
 TGGAGCTGAA GTGCCAGGTG CTGCTGTCCA ACCCGACGTC GGGCTGCTCG TGGCTCTTCC 240  
 AGCCGCGCGG CGCCGCCGCC AGTCCCACCT TCCTCCTATA CCTCTCCCAA AACAAAGCCCA 300  
 AGGCGGCCGA GGGGCTGGAC ACCCAGCGGT TCTCGGGCAA GAGGTGCGGG GACACCTTCG 360  
 TCCTCACCCCT GAGCGACTTC CGCCGAGAGA ACGAGGGCTA CTATTTCTGC TCGGCCCTGA 420  
 GCAACTCCAT CATGTACTTC AGCCACTTCG TGCCGGTCTT CCTGCCAGCG AAGCCCAACA 480  
 CGACGCCAGC GCCGCGACCA CCAACACCGG CGCCACCAT CGCGTCGAG CCCCTGTCCC 540  
 TCGCCCCAGA GCGGTGCCGG CCAGCGGCGG GGGGCGCAGT GCACACGAGG GGGCTGGACT 600  
 TCGCCTGTGA TATCTACATC TGGGCGCCCT TGGCCGGGAC TTGTGGGGTC CTTCTCCTGT 660  
 CACTGGTTAT CACCCTTTAC TGCAACCACA GGAACCGAAG ACGTGTTCG AAATGTCCCC 720  
 GGCCTGTGGT CAAATCGGGA GACAAGCCCA GCCTTTCGGC GAGATACGTC TAACCCTGTG 780  
 CAACAGCCAC TACATTACTT CAAACTGAGA TCCTTCCTTT TGAGGGAGCA AGTCCTTCCC 840  
 TTTCATTTT TCCAGTCTTC CTCCCTGTGT ATTCATTCTC ATGATTATTA TTTAGTGGG 900  
 GCGGGGTGG GAAAGATTAC TTTTCTTTA TGTGTTTGAC GCGAAACAAA ACTAGGTAAA 960  
 ATCTACAGTA CACCACAAGG GTCACAATAC TGTGTGCGC ACATCGCGGT AGGGCGTGGA 1020  
 AAGGGGCAGG CCAGAGCTAC CCGCAGAGTT CTCAGAATCA 1060

## (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 235 amino acids  
 (B) TYPE: amino acid

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ala Leu Pro Val Thr Ala Leu Leu Leu Pro Leu Ala Leu Leu Leu  
1 5 10 15  
His Ala Ala Arg Pro Ser Gln Phe Arg Val Ser Pro Leu Asp Arg Thr  
20 25 30  
Trp Asn Leu Gly Glu Thr Val Glu Leu Lys Cys Gln Val Leu Leu Ser  
35 40 45  
Asn Pro Thr Ser Gly Cys Ser Trp Leu Phe Gln Pro Arg Gly Ala Ala  
50 55 60  
Ala Ser Pro Thr Phe Leu Leu Tyr Leu Ser Gln Asn Lys Pro Lys Ala  
65 70 75 80  
Ala Glu Gly Leu Asp Thr Gln Arg Phe Ser Gly Lys Arg Leu Gly Asp  
85 90 95  
Thr Phe Val Leu Thr Leu Ser Asp Phe Arg Arg Glu Asn Glu Gly Tyr  
100 105 110  
Tyr Phe Cys Ser Ala Leu Ser Asn Ser Ile Met Tyr Phe Ser His Phe  
115 120 125  
Val Pro Val Phe Leu Pro Ala Lys Pro Thr Thr Thr Pro Ala Pro Arg  
130 135 140  
Pro Pro Thr Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg  
145 150 155 160  
Pro Glu Ala Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly  
165 170 175  
Leu Asp Phe Ala Cys Asp Ile Tyr Ile Trp Ala Pro Leu Ala Gly Thr  
180 185 190  
Cys Gly Val Leu Leu Leu Ser Leu Val Ile Thr Leu Tyr Cys Asn His  
195 200 205  
Arg Asn Arg Arg Arg Val Cys Lys Cys Pro Arg Pro Val Val Lys Ser  
210 215 220  
Gly Asp Lys Pro Ser Leu Ser Ala Arg Tyr Val  
225 230 235

40

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCGAAGCTTT CAGTCAGCAT GATAGAAACA

30

50

(2) INFORMATION FOR SEQ ID NO:14:

55

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
 CGCTCTAGAT GTTCAGAGTT TGAGTAAGCC 30

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
 25 CGAAGCTTGG ATCCGAGGAG GTTGGACAAG ATAGAAGAT 39

# Claims

- 30 1. An essentially purified and isolated nucleic acid having a sequence substantially as set forth in Figure 1.
- 35 2. The essentially purified and isolated nucleic acid of claim 1 as contained in CDM8 MC1061/p3-hgp39, as deposited with the American Type Culture Collection and assigned accession number 69050.
3. An essentially purified and isolated nucleic acid comprising a sequence substantially as set forth in Figure 1 from nucleotide residues 160 to 787.
- 40 4. An essentially purified and isolated nucleic acid encoding a protein having a sequence substantially as set forth in Figure 1.
5. An essentially purified and isolated nucleic acid that comprises a nucleotide sequence that encodes a protein having a sequence substantially as set forth in Figure 1 from amino acid residues 47 to 261.
- 45 6. The essentially purified and isolated nucleic acid of claims 3 or 5 further comprising a sequence encoding an extracellular domain of a protein other than a gp39 protein.
7. The essentially purified and isolated nucleic acid of claim 6 further comprising a sequence encoding an extracellular domain of CD8 protein.
- 50 8. The essentially purified and isolated nucleic acid of claim 7 as contained in plasmid CDM7B<sup>-</sup> MC1061/p3-shgp39, as deposited with the American Type Culture Collection and assigned accession number 69049.
- 55 9. An essentially purified and isolated protein having a sequence substantially as set forth in Figure 1.



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10. An essentially purified and isolated protein comprising a sequence substantially as set forth in Figure 1 from amino acid residues 47 to 261.
11. The essentially purified and isolated protein of claim 10 further comprising an extracellular domain of a protein other than a gp39 protein.
12. The essentially purified and isolated protein of claim 11 further comprising an extracellular domain of CD8 protein.
13. The essentially purified and isolated protein of claim 12 as produced by expression of plasmid CDM7B<sup>-</sup> MC1061/p-shgp39, as deposited with the American Type Culture Collection and assigned accession number 69049.
14. An in vitro method of promoting B-cell proliferation comprising exposing activated B-cells to an effective concentration of a soluble gp39 protein.
15. An in vitro method of promoting B-cell proliferation comprising exposing activated B-cells to an effective concentration of at least one protein of claims 10 to 13.
16. An in vitro method of promoting B-cell proliferation comprising exposing B-cells to (i) an effective concentration of a soluble gp39 protein and (ii) a costimulatory substance.
17. The method of claim 16 in which the costimulatory substance is an anti-immunoglobulin antibody.
18. The method of claim 17 in which the costimulatory substance is an antibody directed toward a B-cell antigen.
19. The method of claim 18 in which the B-cell antigen is CD20.
20. Use of a soluble gp39 protein for preparing a pharmaceutical composition suitable for augmenting the immune response of a subject.
21. Use of at least one protein of the claims 10 to 13 for preparing a pharmaceutical composition suitable for augmenting the immune response of a subject.
22. A pharmaceutical composition comprising a therapeutically effective concentration of a soluble gp39 protein in a suitable pharmacological carrier.
23. A pharmaceutical composition comprising a therapeutically effective concentration of at least one protein of claims 10 to 13 in a suitable pharmacological carrier.
24. An in vitro method of promoting B-cell differentiation comprising exposing activated B-cells to an effective concentration of a soluble gp39 protein.
25. An in vitro method of promoting B-cell differentiation comprising exposing activated B-cells to an effective concentration of at least one protein of claims 10 to 13.
26. An in vitro method of promoting B-cell differentiation comprising exposing B-cells to (i) an effective concentration of a soluble gp39 protein and (ii) a costimulatory substance.
27. The method of claim 26 in which the costimulatory substance is an anti-immunoglobulin antibody.
28. The method of claim 26 in which the costimulatory substance is an antibody directed toward a B-cell antigen.
29. The method of claim 26 in which the B-cell antigen is CD20.

30. An in vitro method of promoting the proliferation of cells that bear CD40 comprising exposing the cells to an effective concentration of a soluble gp39 protein.
- 5 31. An in vitro method of promoting the differentiation of cells that bear CD40 comprising exposing the cells to an effective concentration of a soluble gp39 protein.
32. The method of claim 30 or 31 in which the cells are sarcoma cells.
- 10 33. Use of a soluble gp39 protein for preparing a pharmaceutical composition suitable for promoting B-cell proliferation.
34. Use of at least one protein of claims 10 to 13 for preparing a pharmaceutical composition suitable for promoting B-cell proliferation.
- 15 35. The use of claim 33 or 34, wherein additionally a costimulatory substance is applied.
36. The use of claim 33 or 34 in which the costimulatory substance is an anti-immunoglobulin antibody.
- 20 37. The use of claim 36 in which the costimulatory substance is an antibody directed toward a B-cell antigen.
38. The use of claim 37 in which the B-cell antigen is CD20.
- 25 39. Use of a soluble gp39 protein for preparing a pharmaceutical composition suitable for promoting B-cell differentiation.
40. Use of at least one protein of claims 10 to 13 for preparing a pharmaceutical composition suitable for promoting B-cell differentiation.
- 30 41. The use of claim 39 or 40, wherein additionally a costimulatory substance is applied.
42. The use of claim 41 in which the costimulatory substance is an anti-immunoglobulin antibody.
- 35 43. The use of claim 41 in which the costimulatory substance is an antibody directed toward a B-cell antigen.
44. The use of claim 41 in which the B-cell antigen is CD20.
- 40 45. Use of a soluble gp39 protein for preparing a pharmaceutical composition suitable for promoting the proliferation and/or the differentiation of cells that bear CD40.
46. The use of claim 45 in which the cells are sarcoma cells.

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1      MetIleGluThrTyrAsnGlnThrSerPro
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11     ArgSerAlaAlaThrGlyLeuProIleSerMetLysIlePheMetTyrLeuLeu
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29     ThrValPheLeuIleThrGlnMetIleGlySerAlaLeuPheAlaValTyrLeu
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Figure 1A

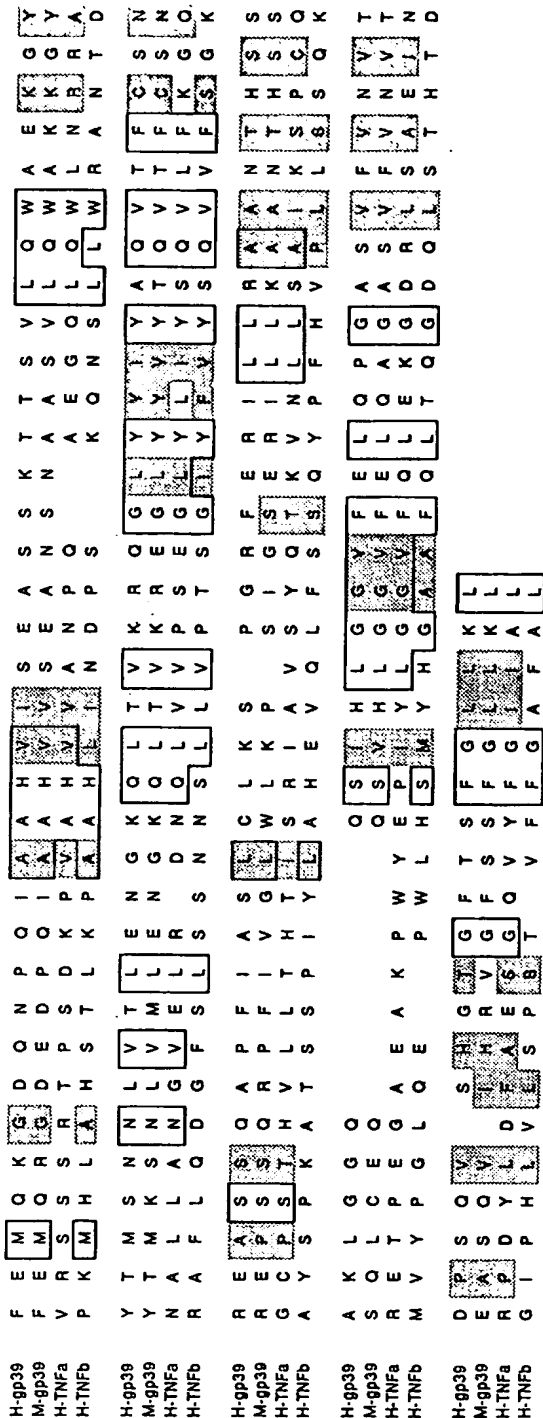


Figure 1B

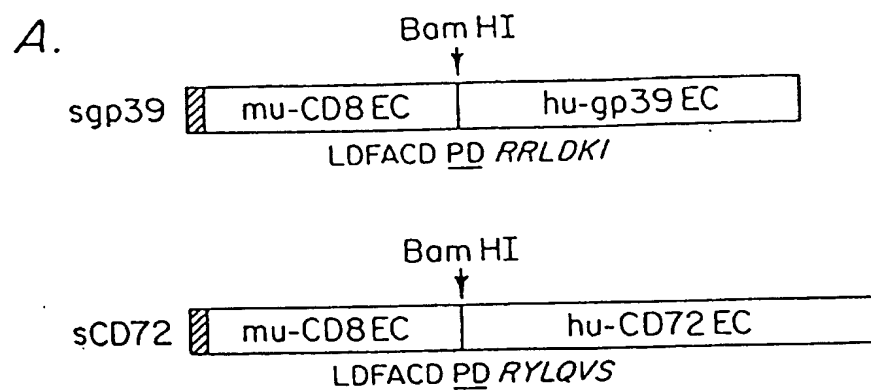


Figure 2A

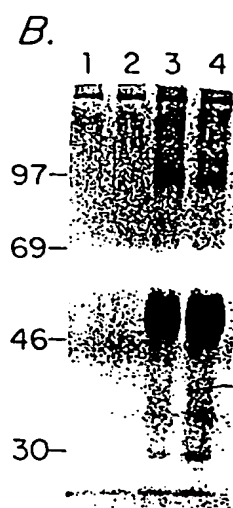


Figure 2B

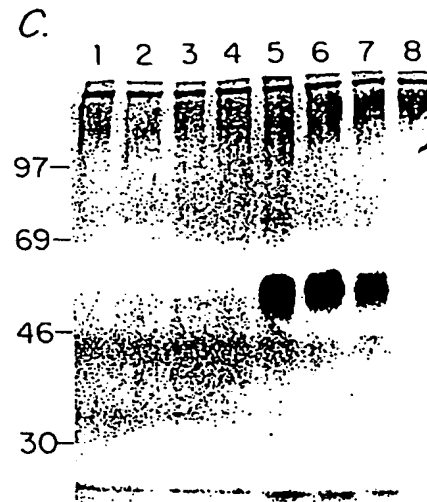


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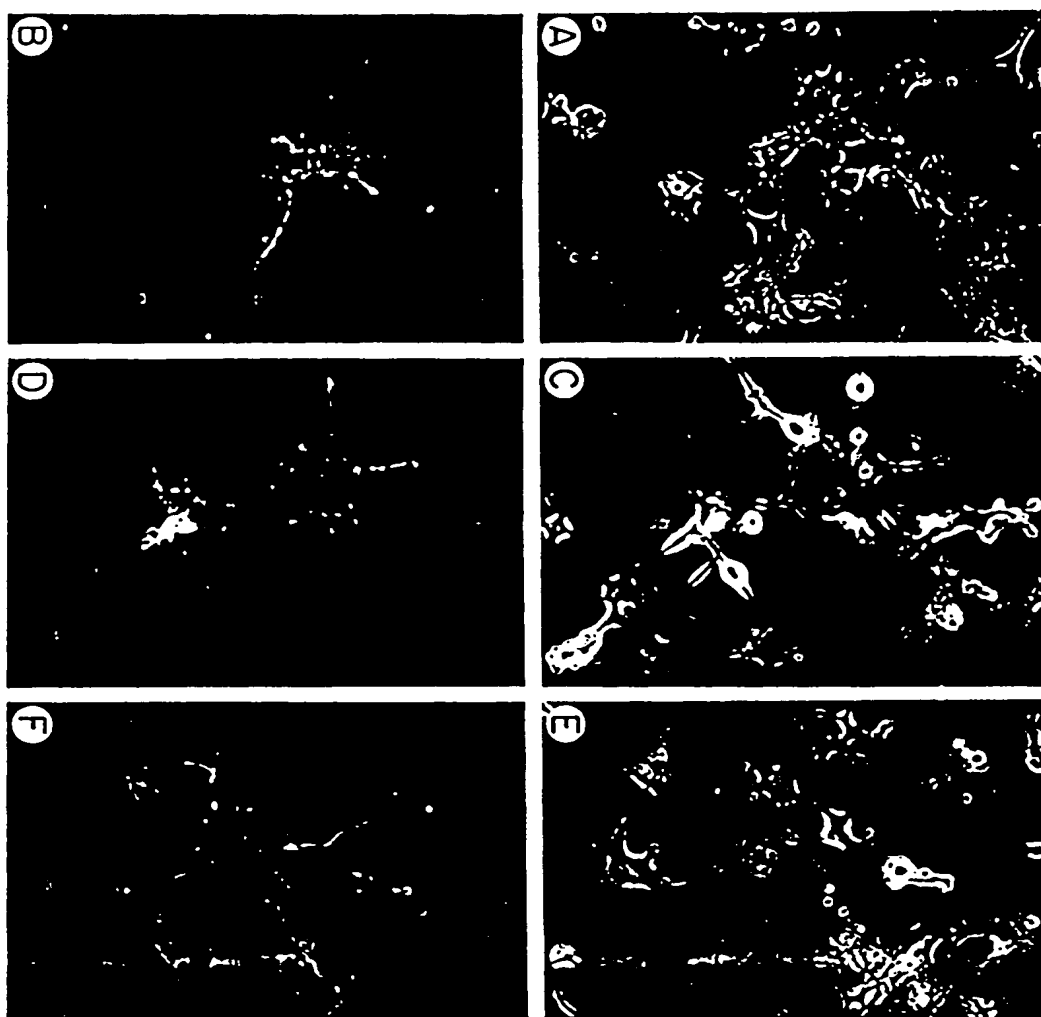


Figure 3



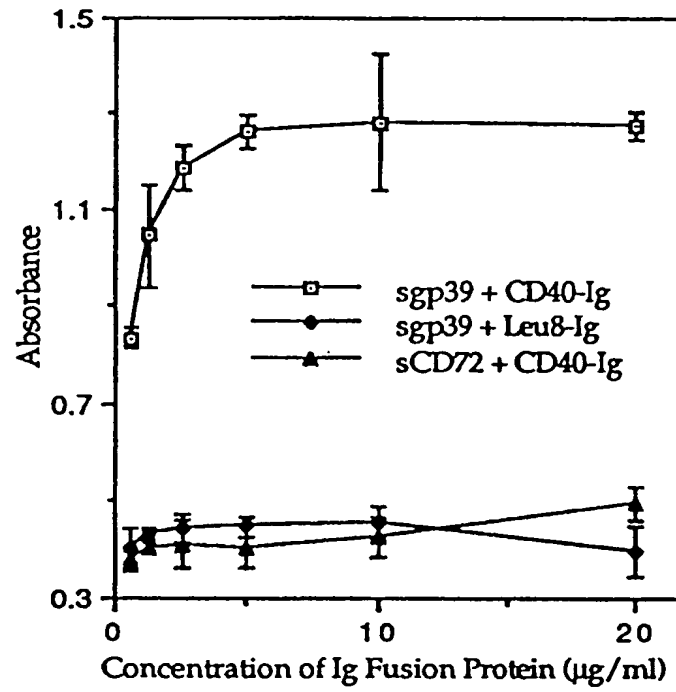


Figure 4

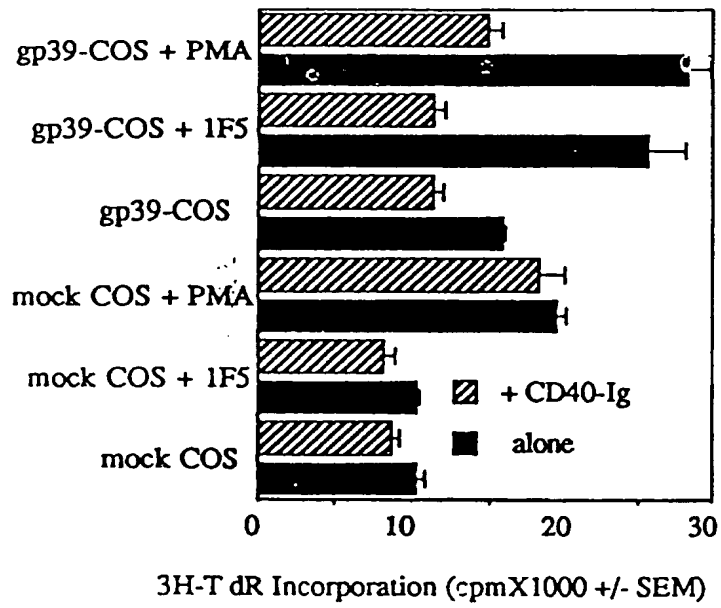


Figure 5

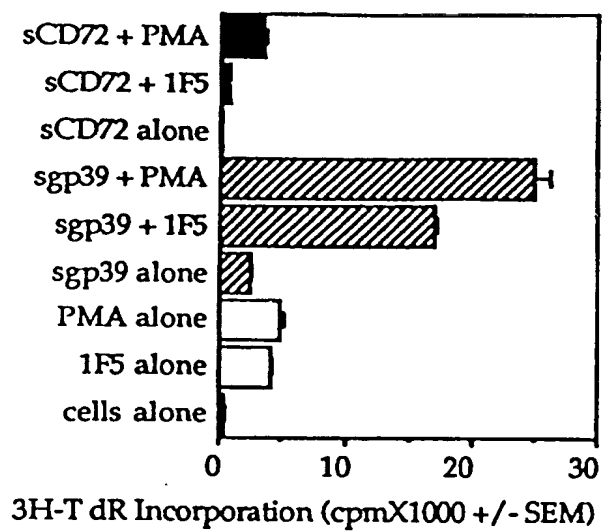


Figure 6

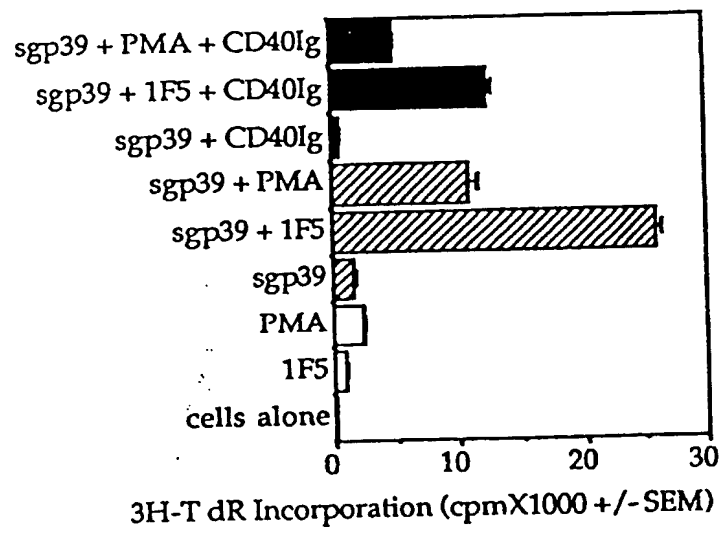


Figure 7A

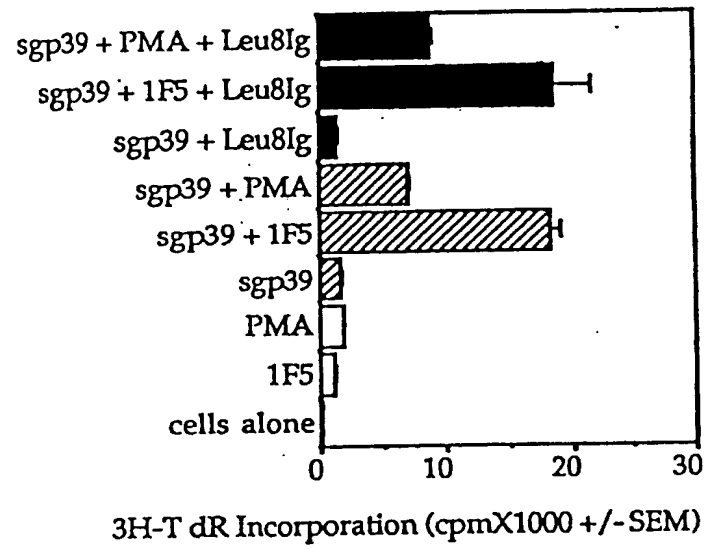


Figure 7B

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Figure 8

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 201 VITLYCNHRN RRRVCKCPAP VVKSGDKPSL SARVY\*

Figure 9